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Rabies Virus Inactivation by Binary Ethylenimine: New Method for Inactivated Vaccine Production

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The inactivation dynamics of rabies virus (PV strain) by binary ethylenimine, and the immunogenic properties and the stability of the vaccines prepared using this agent, were studied. Binary ethylenimine at a final concentration of 0.01 M was prepared with 2-bromoethylamine hydrobromide in alkaline solutions, either separately from or in suspensions of rabies virus propagated in BHK cells. The infectivity of virus suspensions containing more than 10^8 plaque-forming units per 0.1 ml was inactivated in 2 h when the inactivating agent was prepared before its addition to the suspensions, and in 3 h when prepared directly in the suspensions. Liquid vaccines prepared in this manner and stored at different temperatures maintained potency for 1 month at 37°C and for 6 months at 4°C and 22 to 25°C. Lyophilized vaccine maintained its potency for 6 months at the three temperatures. The inactivated vaccine mixed with aluminum or oil adjuvant at high dilutions protected guinea pigs against challenge. This safer procedure for rabies virus inactivation offers promise for the production of effective vaccines for the immunization of dogs and cattle.

Effective rabies vaccines have been prepared by using beta-propiolactone, acetylethylenimine, and ethylenimine (EI) to inactivate the virus (10, 11, 20). These chemical agents act on the nucleic acid, with little or no effect on the immunogenic properties of the viral protein coat, but they are potentially dangerous when used in large volumes. Since beta-propiolactone is a vesicant (18) and the aziridines (EI and derivatives) are highly toxic (1), special precautions have to be taken when they are used in the pure state (4). Accidents have been reported with the use of acetylethylenimine (1).

By cyclization of 2-bromoethylamine hydrobromide (BEA) in alkaline solutions, Bahnemann inactivated foot-and-mouth disease, vesicular stomatitis, and infectious bovine rhinotracheitis viruses (1, 2). The product of this synthesis, designated binary ethylenimine (BEI) to differentiate it from pure EI, is less hazardous than is liquid EI for use in inactivation procedures.

The present report deals with studies performed on inactivation of rabies virus by BEI, and on the immunogenic properties and stability of the vaccine prepared by this inactivation procedure.

MATERIALS AND METHODS

Virus. The PV strain of rabies virus was propagated in BHK-21 C_{13} cell cultures, with some modification of the techniques described earlier (9, 10). Briefly, cell cultures in Blake (1×10^8 to 1.2×10^8 cells per bottle) and roller (2×10^8 cells per bottle) flasks were infected at a multiplicity of 1 to 5 plaque-forming units of virus per cell. The maintenance medium used was that described by Capstick (3) with 0.3% bovine albumin; HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) at 0.02 M or TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] at 0.0275 M was included to stabilize the pH (7.4 to 7.6). The culture fluid was harvested 3 and 6 days after infection of the cells.

Viral inactivation. By using BEA (Eastman Kodak Co., Rochester, N.Y.), BEI at a final concentration of 0.01 M was prepared separately from and directly in the virus suspensions, as described by Bahnemann (1).

Viral inactivation was performed at 37°C in a water bath, and samples were taken at periodic intervals for titration by the plaquing technique (14). The inactivation was stopped with sodium thiosulfate (10).

Vaccine production and control. Once the inactivation conditions had been determined, viral suspensions were inactivated with BEI prepared directly in the suspensions and incubated for 6 h at 37°C, as described above. The pH was adjusted to between 7.4 and 7.6 with sodium bicarbonate, and the stabilizer (16) was added. In some cases, aluminum hydroxide (final concentration of aluminum oxide, 1.2%) and oil (nine parts of Arlacel to one part Bayol; Esso International, New York) were added to the vaccine as adjuvants.

The sterility and safety of every lot of vaccine were tested as described earlier (10). The potency was checked by the modified National Institutes of Health (NIH) test (16), using reference vaccine lot 7 from this

center, the antigenic value minimum requirement of which was 0.4.

The potency of the vaccines with adjuvant was checked by the Koprowski test (8) by immunizing groups of guinea pigs with fivefold dilutions of the vaccines. These and unvaccinated guinea pigs were challenged with a dilution (1:500 of a virus suspension titrating $10^{6.7}$ 50% lethal doses per 0.03 ml in mice) of DR19 virus (5), which killed 75% of the controls.

RESULTS

Figure 1 presents the inactivation kinetics of PV virus by BEI. The infectivity of virus suspensions containing over 10^8 PFU/0.1 ml was inactivated in 2 h when the BEI was prepared separately from the virus suspensions and in 3 h when it was prepared in the suspensions.

During this study, we prepared 57 batches of vaccine, all of which passed the sterility, safety, and potency tests. These vaccines passed the NIH test with antigenic values between 4 and 40, results which were 10 to 100 times higher than the minimum requirement for the reference vaccine used.

A batch of vaccine was divided into two portions; one was kept in the liquid state, and the other was lyophilized. Both were further divided into three fractions, which were kept at 4°C, at room temperature (22 to 25°C), or at 37°C for 6 months. The results of the potency tests performed on each fraction during this period are shown in Table 1.

Table 2 presents the results of the Koprowski potency test performed with the vaccine either (i) alone, (ii) supplemented with aluminum hydroxide, or (iii) supplemented with oil adjuvant.

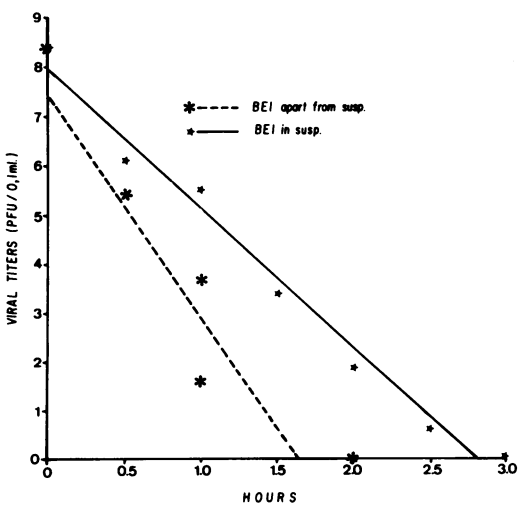

FIG. 1. *PV rabies virus inactivation by BEI at 37°C.*

TABLE 1. *Stability of a rabies tissue culture vaccine inactivated with BEI*

Vaccine	Storage temp (°C)	Stability on day of storage[a]: 0	13	28	90	180
Liquid	4	4.7	14.7	3.8	2.9	4.9
	22–25		9.9	5.5	0.8	0.5
	37		7.5	1.6	0.1	<0.1
Lyophilized	4	5.0	5.4	2.2	1.4	4.7
	22–25		8.0	1.3	1.1	5.6
	37		7.5	1.0	2.3	1.9

[a] Each value is based on antigenic value, NIH potency test. Minimum requirement = 0.4.

TABLE 2. *Dilution of an inactivated rabies tissue culture vaccine protecting 50% of the vaccinated guinea pigs*[a]

Vaccine	ED_{50}	$ED_{50} \pm 2$ SE
Alone	13,975	3,390–57,604
Oil adjuvant	7,341	3,283–16,416
Aluminum adjuvant	4,530	1,135–18,080

[a] 75% of the unvaccinated controls died of rabies. Each value represents reciprocal dilution value. Calculated by Spearman-Karber. SE, Standard error. ED_{50}, 50% effective dose.

No statistical differences were found in the 50% effective doses of the three vaccines. Furthermore, 11 other batches of vaccine with adjuvant were examined by the same test, with satisfactory results.

DISCUSSION

The present findings demonstrate that rabies tissue culture vaccine inactivated with BEI is very potent and stable (Table 1). The fact that this vaccine was still potent after 1 month at 37°C in the liquid form, more stringent conditions than used previously with EI-inactivated vaccine (10), favors its application in the field. As stated in a previous publication (10), the individual variations of antigenic values may be inherent in the potency test.

Different chemical and physical agents have been used to inactivate rabies virus for vaccine production. At present, phenol (15), beta-propiolactone (7, 12, 13), and ultraviolet light (6) are the most widely used for the purpose.

EI is a very stable and inexpensive reagent, but like other EI derivatives (4) and beta-propiolactone, it requires very careful handling. Phenol is not a very satisfactory inactivating agent for rabies virus, because it acts on all of the viral proteins, including the envelope glycoprotein. This antigen is responsible for stimulating the production of viral neutralizing antibodies (21). A limitation of ultraviolet inactivation

is the Dill apparatus required, which is expensive and difficult to maintain. Recently, it was also shown that EI readily inactivates rabies virus propagated in tissue culture (10) or in suckling mouse brain (unpublished data).

In contrast, BEI has the advantages of having the same stability and low price as EI, and of being less hazardous to handle. Protection of bare skin against contact toxicity is still indicated, however, when large volumes of 0.1 M BEI solutions are handled (1).

BEI has been used to inactivate several animal viruses (1, 2, 19) for vaccine or antigen production. It has inactivated more than 6 logs of foot-and-mouth disease virus in 40 min (1) and 8 logs of pseudorabies virus in 6 h (19). It also inactivates rabies virus very efficiently, in a first-order reaction (Fig. 1). In our experience, it inactivated the infectivity of more than 8 logs of virus in 2 h when BEI was prepared before its addition to the viral suspensions, and in 3 h when prepared directly in the suspensions.

On the basis of the results of the NIH potency tests included in this report (10 to 100 times higher than the minimum requirements), the immunization of dogs with this vaccine should be expected to provide them a good immunity. The same type of vaccine inactivated with EI, having an antigenic value 20 times as great as the minimum requirement for the NIH test, has protected 89% of the vaccinated dogs 3 years after vaccination (O. P. Larghi, V. L. Savy, E. Nebel, and A. Rodriguez, Rev. Arg. Microbiol., in press). Sikes et al. (17) protected 100% of vaccinated dogs with a purified vaccine whose antigenic value was 10 times as great as that requirement. The findings with vaccines in adjuvant (Table 2) suggest their application for rabies immunization of cattle. Studies on the duration of immunity afforded to this species are in progress at our laboratories.

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